APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2007, p. 2735–2743 Vol. 73, No. 8
0099-2240/07/$08.00+0 doi:10.1128/AEM.02829-06

Gene Targeting in Gram-Negative Bacteria by Use of a Mobile Group II Intron ("Targetron") Expressed from a Broad-Host-Range Vector[▽]

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Received 5 December 2006/Accepted 16 February 2007

Mobile group II introns ("targetrons") can be programmed for insertion into virtually any desired DNA target with high frequency and specificity. Here, we show that targetrons expressed via an *m*-toluic acid-inducible promoter from a broad-host-range vector containing an RK2 minireplicon can be used for efficient gene targeting in a variety of gram-negative bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. Targetrons expressed from donor plasmids introduced by electroporation or conjugation yielded targeted disruptions at frequencies of 1 to 58% of screened colonies in the *E. coli lacZ*, *P. aeruginosa pqsA* and *pqsH*, and *A. tumefaciens aopB* and *chvI* genes. The development of this broad-host-range system for targetron expression should facilitate gene targeting in many bacteria.

Mobile group II introns ("targetrons") have been used for targeted gene disruption and site-specific DNA insertion in diverse gram-negative and gram-positive bacteria, including *Escherichia coli* (21, 30), *Shigella flexneri* (21), *Salmonella enterica* serovar Typhimurium (21), *Lactococcus lactis* (11), *Clostridium perfringens* (3), and *Staphylococcus aureus* (45). Group II introns are useful for gene targeting because they can be programmed for insertion into virtually any desired DNA target with high frequency and specificity (22, 30). This ability derives from their unique DNA integration mechanism, called retrohoming, in which the intron RNA is inserted via reverse splicing directly into a DNA target site and is then reverse transcribed by the intron-encoded protein (IEP) (reviewed in reference 23). Retrohoming is mediated by a ribonucleoprotein (RNP) particle that is formed during RNA splicing and contains the IEP and excised intron lariat RNA. RNPs initiate mobility by using both the IEP and base pairing of the intron RNA to recognize DNA target sequences, with the latter contributing most of the target specificity (10, 16, 17, 41). Consequently, it is possible to retarget group II introns for insertion into preselected sites simply by modifying the base-pairing sequences in the intron RNA (16, 22, 28, 30).

DNA target site interactions for the *L. lactis* Ll.LtrB group II intron used in the present work are shown in Fig. 1A (16, 28, 30, 41). The DNA target site positions recognized by base pairing of the intron RNA span positions −12 to +2 from the intron insertion site and are denoted intron-binding sites 1 and 2 (IBS1 and IBS2) in the 5′ exon (E1) and δ′ in the 3′ exon (E2). The complementary intron RNA sequences are located in two different RNA stem loops and are denoted exon-binding sites 1 and 2 (EBS1 and EBS2) and δ sequences (sequences adjacent to EBS1). The IEP (LtrA protein) recognizes additional positions in the distal 5′ exon and 3′ exon regions of the DNA target site, interactions that are important for local DNA melting and bottom-strand cleavage for generating the primer for reverse transcription of the inserted intron RNA (key positions recognized by the IEP are underlined in Fig. 1A) (22, 23). The Ll.LtrB intron is retargeted with the aid of a computer algorithm that scans the target sequence for the best matches to the positions recognized by the IEP and then designs PCR primers for modifying the intron's EBS1, EBS2, and δ sequences to base pair optimally to the IBS1, IBS2, and δ′ sequences in the DNA target site (30). The positions recognized by the IEP are sufficiently few and flexible that the algorithm generally identifies multiple rank order target sites in any gene. In bacteria, targetrons designed using the algorithm are commonly inserted into chromosomal target sites at frequencies of 1 to 100% of colonies without selection, and insertions can be detected either by colony PCR screening or by using a genetic marker inserted in the intron (30, 47). Targetrons have been used in bacteria to obtain targeted gene disruptions, including conditional disruptions (11, 21, 30, 45), insertion ("knocking in") of cargo genes at desired chromosomal locations (11, 34), introduction of point mutations by making a targeted double-strand break that stimulates homologous recombination with a cotransformed DNA (21), and generation of whole-genome knockout libraries (46, 47).

In principle, targetrons can be used in any bacterium in which they can be introduced and expressed from a donor plasmid. The *E. coli* donor plasmids pACD2 and pACD3 employ a T7*lac* promoter to transcribe the Ll.LtrB targetron and are used in host strains that express T7 RNA polymerase from a λ DE3 prophage or a cotransformed plasmid (21). In *C. perfringens*, *L. lactis*, and *S. aureus*, targetrons have been expressed from established plasmid vectors for those organisms by using a variety of constitutive or inducible promoters (an alpha-toxin gene promoter for *C. perfringens* and nisin-inducible and cadmium-inducible promoters for *L. lactis* and *S. aureus*, respectively) (3, 11, 45). The extent to which these existing donor plasmids and promoters can function in other hosts is unknown, and consequently, targetrons have generally been cloned into a new expression vector for each organism.

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▽ Published ahead of print on 23 February 2007.

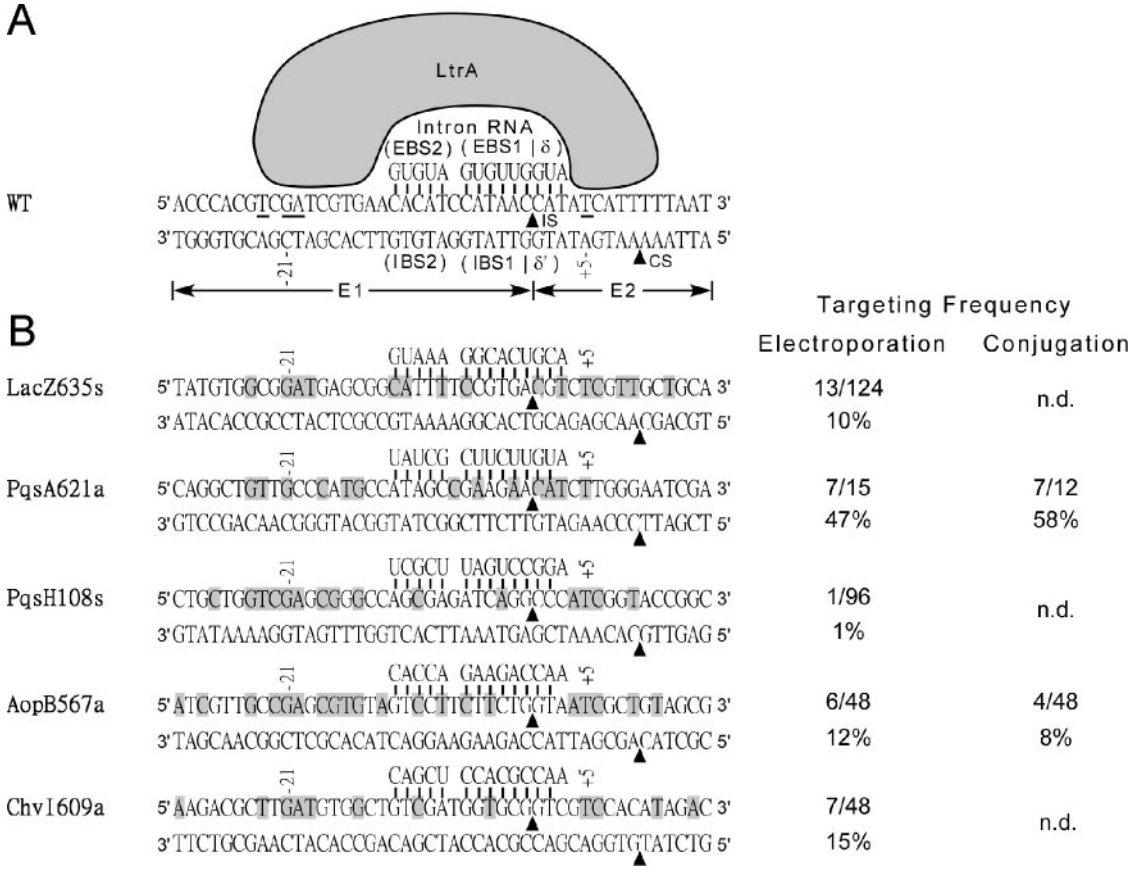

FIG. 1. DNA target site interactions for the wild-type Ll.LtrB intron and targetron derivatives used in the present work. (A) DNA target site interactions for the wild-type (WT) Ll.LtrB intron. The DNA target site is recognized by an RNP containing the IEP (LtrA protein) and excised intron lariat RNA, with both the protein and base pairing of the intron RNA used to recognize DNA target sequences. Key bases recognized by the IEP (underlined) include T −23, G −21, and A −20 in the 5′ exon (E1) and T +5 in the 3′ exon (E2). Additional nucleotide residues (not highlighted) also contribute directly or indirectly to IEP recognition (30). The intron RNA's EBS2, EBS1, and δ sequences base pair with IBS2, IBS1, and δ′ sequences located between DNA target site positions −12 and +2. (B) DNA target site sequences and intron RNA/DNA target site base pairings for targetrons LacZ635s, PqsA621a, PqsH108s, AopB567a, and ChvI609a, designed for insertion into the *E. coli lacZ*, *P. aeruginosa pqsA* and *pqsH*, and *A. tumefaciens aopB* and *chvI* genes, respectively. Targetrons are named by the nucleotide position 5′ to their insertion site in the target gene's coding sequence, followed by "s" or "a," indicating sense or antisense strands, respectively. DNA target sequences are shown from positions −30 to +15 from the intron insertion site. Nucleotide residues in the DNA target sites that match those in the wild-type Ll.LtrB intron target site are highlighted in gray in the top strand. Targeting frequencies for targetrons expressed from pBL1 introduced via electroporation or conjugation are summarized to the right. Integration frequencies are expressed as the fraction of colonies analyzed, with the percentage indicated beneath. The intron insertion site in the top strand (IS) and the bottom-strand cleavage site (CS) are indicated by arrowheads. n.d., not determined.

Here, we sought to overcome this limitation by expressing targetrons from a derivative of the broad-host-range expression vector pJB866 (2). This vector contains an *m*-toluic acid-inducible promoter and a mini-RK2 replicon, which has been shown to support replication in a variety of gram-negative bacteria (37). We show that targetrons expressed from this vector can be used for efficient gene targeting in *E. coli*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. Further, we show that targetrons introduced into *P. aeruginosa* and *A. tumefaciens* by conjugation function as efficiently for gene targeting as those introduced by electroporation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains DH5α [F^- φ80*dlacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-], HMS174(DE3) [F^- *recA1 hsdR* (r_{K12}^- m_{K12}^+) Rif^{r}λDE3], and S17.1 (*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7*) (40) were grown in Luria-Bertani (LB) medium. Tetracycline and chloramphenicol were used at 25 μg/ml.

P. aeruginosa strain PAO1 (obtained from Marvin Whiteley, University of Texas at Austin) was grown in LB broth or on LB or brain heart infusion (BHI) agar plates. Tetracycline was used at 80 μg/ml.

A. tumefaciens C58 (obtained from Eugene Nester, University of Washington) was grown at 30°C in mannitol glutamate/L broth (MG/L; 1:1 [vol/vol]) (13) or *Agrobacterium* broth minimal medium (24). Tetracycline was used at 2 μg/ml for plates and 10 μg/ml for liquid culture.

Recombinant plasmids. Targetron donor plasmid pBL1 (Fig. 2A) contains the targetron cassette of pACD2X (3-kb HindIII/XhoI fragment) (36) cloned downstream of the *m*-toluic acid-inducible promoter (*Pm*) between the HindIII and XhoI sites of pJB866 (2). The targetron cassette consists of a 0.9-kb Ll.LtrB-ΔORF intron and flanking exons, with the LtrA open reading frame (ORF) cloned downstream of the 3′ exon (36).

Retargeting the Ll.LtrB intron for insertion into *P. aeruginosa* and *A. tumefaciens* genes. The Ll.LtrB targetron was retargeted for insertion into *P. aeruginosa* and *A. tumefaciens* genes by using a computer algorithm that identifies

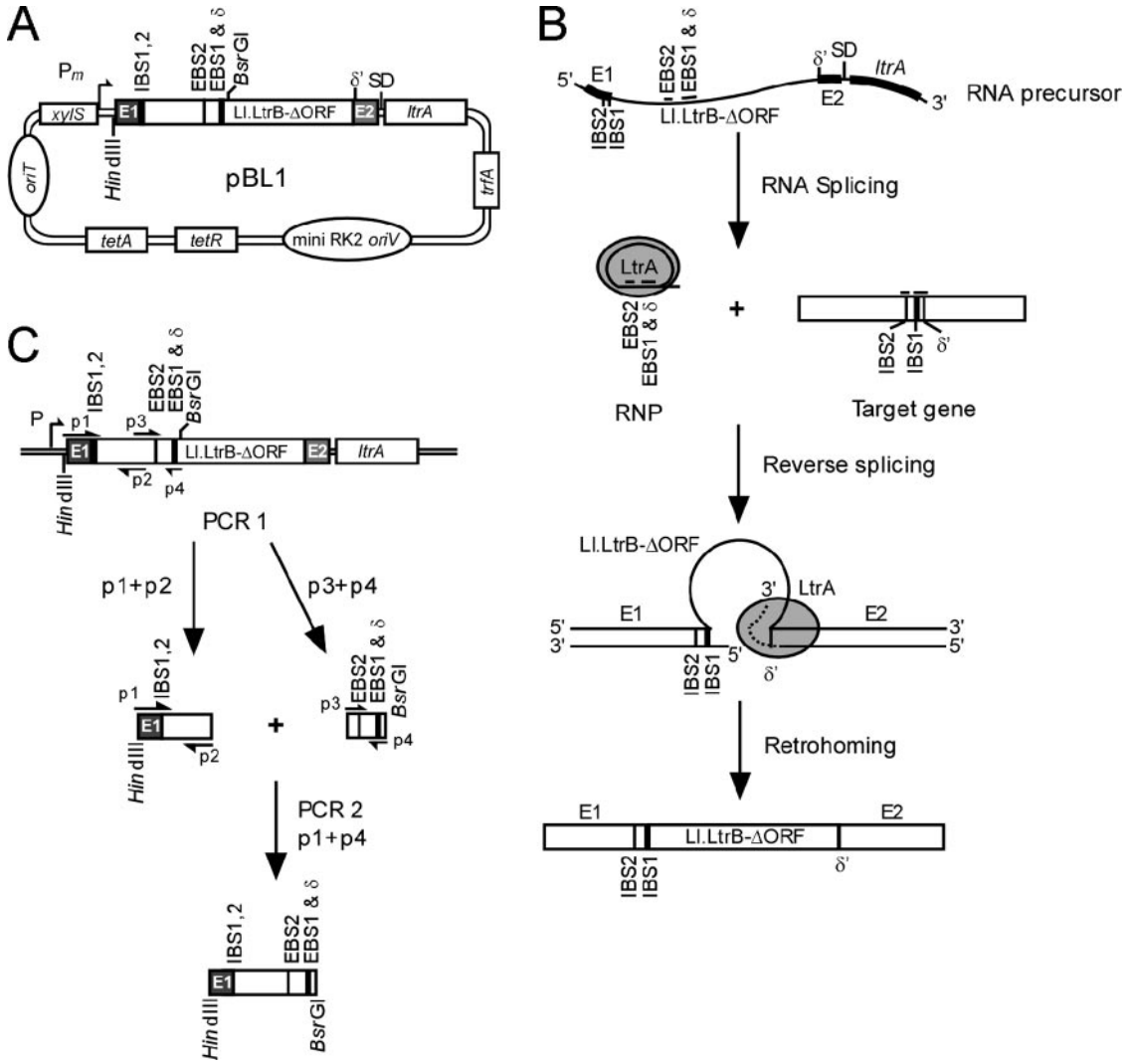

FIG. 2. Broad-host-range targetron expression plasmid pBL1 used for gene targeting in gram-negative bacteria and steps in gene targeting. (A) Plasmid pBL1 is a derivative of the broad-host-range expression vector pJB866 (2) and employs an *m*-toluic acid-inducible promoter (*Pm*) regulated by transcriptional activator XylS to express a targetron cassette containing the Ll.LtrB-ΔORF intron and short flanking 5′ and 3′ exon sequences (E1 and E2, respectively) plus the LtrA ORF downstream of E2. The vector contains a mini-RK2 replicon, consisting of the RK2 vegetative replication origin (*oriV*) and *trfA*, encoding a protein essential for initiation of replication at *oriV*. The vector also contains a conjugation transfer origin (*oriT*); *tetA*, which confers tetracycline resistance; and *tetR*, which represses *tetA* in the absence of tetracycline. SD is the Shine-Dalgarno sequence used for translation of the LtrA protein. (B) Targetron expression and DNA integration. The targetron is expressed from pBL1 as a precursor RNA containing the Ll.LtrB-ΔORF intron and flanking exon sequences, followed by the LtrA coding sequence, with its own Shine-Dalgarno sequence. The LtrA protein binds to the intron in the precursor RNA and promotes its splicing by stabilizing the catalytically active RNA structure. Base pairing between the EBS1 and EBS2 sequences in the intron and the IBS1 and IBS2 sequences in the 5′ exon of the precursor RNA is also required for efficient RNA splicing. After RNA splicing, RNPs initiate DNA integration by recognizing DNA target sites, using both the IEP and base pairing of the intron RNA's EBS2, EBS1, and δ sequences to IBS2, IBS1, and δ′ in the DNA target site (see Fig. 1A). The intron RNA is then inserted ("reverse spliced") into the intron insertion site (IS) in the top strand of the DNA target site, while the LtrA protein cleaves the bottom strand between positions +9 and +10 and uses the 3′ end at the cleavage site as a primer for reverse transcription of the inserted intron RNA (23). The resulting intron cDNA is integrated by host cell DNA repair mechanisms (43). (C) Two-step PCR procedure used to retarget the Ll.LtrB intron for insertion into preselected target sites. The PCRs modify the EBS1, EBS2, and δ sequences in the Ll.LtrB-ΔORF intron to base pair to the IBS1, IBS2, and δ′ sequences in the DNA target site and also modify IBS1 and IBS2 in E1 of the donor plasmid to base pair to the intron's retargeted EBS1 and EBS2 sequences for efficient RNA splicing. The first PCR step (PCR1) uses primers p1 plus p2 and p3 plus p4. In the second PCR step (PCR2), the gel-purified products of the first step are mixed and amplified with the outside primers p1 and p4 to yield a 353-bp product, which is digested with HindIII and BsrGI and swapped for the corresponding fragment of the donor plasmid.

potential Ll.LtrB intron insertion sites and designs PCR primers for modifying the intron RNA to base pair optimally to those sites (30). First, single target sites in the *P. aeruginosa pqsA* and *pqsH* genes, which encode enzymes involved in synthesis of a quinolone signaling molecule, and the *A. tumefaciens aopB* and *chvI* genes, which encode an outer membrane protein and virulence factor, respectively, were chosen from among potential target sites identified by the algorithm for each gene. Donor plasmids were then constructed by a two-step PCR, using primers designed by the algorithm to modify the intron's EBS1, EBS2, and δ sequences to base pair to DNA target site sequences IBS2 (5′ exon positions −12 to −8), IBS1 (5′ exon positions −6 to −1), and δ′ (3′ exon

positions +1 and +2) (21). In the first step, overlapping segments of the donor plasmid were amplified by two PCRs, one using primers p1 and p2 and the other using primers p3 and p4 (Fig. 2C). p1 contains 5′ exon positions −25 to +18, with modifications at positions −12 to −1 to make IBS1 and IBS2 in the donor plasmid complementary to the retargeted EBS1 and EBS2 sequences for efficient splicing, and has positions −25 to −13 changed to ATAATTATCCTTA to minimize self-targeting (30); p2 corresponds to intron positions +192 to +219 (5′-CGAAATTAGAAACTTGCGTTCAGTAAAC); p3 corresponds to intron positions +198 to +246, with modifications at positions +223 to +227 to make the EBS2 sequence complementary to IBS2 in the DNA target site; and p4 corresponds to intron positions +259 to +318, with modifications at intron positions +276 to +285 to make the EBS1 and δ sequences complementary to the DNA target's IBS1 and δ′ sequences, respectively. In the second step, the two gel-purified PCR products from the first step were mixed and amplified with the outside primers p1 and p4 to generate a 353-bp PCR product containing sequences corresponding to the 5′ exon and 5′ end of the intron (nucleotide positions E1 −25 to I +318). The final PCR product was purified in a 0.8% agarose gel, digested with BsrGI and HindIII, and swapped for the corresponding fragment of the donor plasmid.

Gene targeting in *E. coli*. *E. coli* HMS174(DE3) was transformed with pBL1 containing targetron LacZ635s, which inserts site specifically into the *lacZ* gene. The cells were grown overnight at 37°C, induced with 2 mM *m*-toluic acid for 1 h at 37°C, and plated on LB agar with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to score *lacZ* disruptants by blue/white screening.

Gene targeting in *P. aeruginosa*. *P. aeruginosa* PAO1, containing targetron donor plasmids introduced by electroporation (4) or conjugation (2), was grown in LB medium plus tetracycline until early log phase (optical density at 595 nm [OD_{595}] = 0.3 to 0.4) and then induced with 2 mM *m*-toluic acid overnight at 30°C. After induction, cells were plated on LB agar, and the plates were incubated at 37°C. For conjugation, *E. coli* S17.1, containing the targetron donor plasmid, and *P. aeruginosa* PAO1 were grown separately to early log phase (OD_{595} = 0.3 to 0.4) and then harvested and mixed at a ratio of 10:1 in 20 ml LB medium. The mixed cells were collected by filtration on a 25-mm-diameter membrane filter (0.45-μm pore size; Millipore, Billerica, MA), and the filter was placed on an LB agar plate. After incubation at 30°C for 3 h, the filter was transferred to a 15-ml conical tube, and mating was disrupted by vigorous shaking in 5 ml of LB, followed by incubation for 3 h at 30°C. The culture was then serially diluted and plated on LB agar containing both tetracycline to select for *P. aeruginosa* containing the conjugated plasmid and chloramphenicol (25 μg/ml). Chloramphenicol kills *E. coli* S17.1 but not *P. aeruginosa* PAO1, which has high intrinsic chloramphenicol resistance.

Gene targeting in *A. tumefaciens*. *A. tumefaciens* C58, containing targetron donor plasmids introduced by electroporation (25) or conjugation (2), was grown in tetracycline-containing MG/L medium (*aopB* targeting) or *Agrobacterium* broth minimal medium (*chvI* targeting) at 30°C until early log phase (OD_{595} = 0.3 to 0.4) and then induced with 5 mM *m*-toluic acid for 3 h at the same temperature. After induction, cells were plated on the same medium, and the plates were incubated at 30°C. Conjugation was as described above for *P. aeruginosa*, except that conjugants were selected by plating them on MG/L agar containing tetracycline plus ampicillin (100 μg/ml), which kills *E. coli* S17.1 but not *A. tumefaciens* C58.

Detection of targetron integration by colony PCR. Targetron integration in the *lacZ*, *pqsA*, *pqsH*, *aopB*, and *chvI* genes was detected by colony PCR (18), using primers, referred to generically as p_f and p_r, that flank the insertion site in the target gene. Primers were as follows: for *lacZ*, 5′-ATCCTGCAGCGGATAACAATTTCACACAG and 5′-ATCCTGCAGACATGGCCTGCCCGG; for *pqsA*, 5′-ATTGGCCAACCTGACCGAGG and 5′-GCCGAGGCTCCGCTGAACC; for *pqsH*, 5′-GAGCAACGGATGACCGTTCTTATCC and 5′-GACATCAGCATCGAACCGTCG; for *aopB*, 5′-GCCGACGCCGTAAATGAG and 5′-GACCGAGTGGTCGTCAAAACC; and for *chvI*, 5′-CTGACAGTGACTGAATTCCTCATCC and 5′-GCGGTCCCTTCTGCTTGAG.

Curing of the targetron donor plasmid. The targetron donor plasmid was cured by growing cells overnight in the absence of tetracycline (LB medium at 37°C for *P. aeruginosa* and MG/L medium at 30°C for *A. tumefaciens*) and then plating them on LB or MG/L agar, again without tetracycline. Colonies that had lost the donor plasmid (17 to 25% for the PqsA and PqsH donor plasmids in *P. aeruginosa* and 100% for the AopB donor plasmid in *A. tumefaciens*) were identified by colony PCR and sensitivity to tetracycline.

Southern hybridization. After curing of the targetron donor plasmid, cells were grown until late log phase, and DNA was isolated by using a bacterial genomic DNA prep kit (QIAGEN, Valencia, CA). Southern hybridization was done as described previously (30), using a ^{32}P-labeled intron probe generated by PCR of intron donor plasmid pACD2X (36), with primers 5′-TCTTGCAAGGGTACGGAGTA and 5′-GTAGGGAGGTACCGCCTTGTTC. The probe was labeled with [α-^{32}P]dTTP (3,000 Ci/mmol; PerkinElmer, Wellesley, MA), using a High Prime DNA labeling kit (Roche Diagnostics, Indianapolis, IN).

RESULTS

Construction of broad-host-range targetron donor plasmid pBL1. For gene targeting in gram-negative bacteria, we constructed the broad-host-range targetron donor plasmid pBL1 (Fig. 2A), in which the targetron is cloned downstream of an *m*-toluic acid-inducible promoter (*Pm*) in the broad-host-range vector pJB866 (2). Transcription from the *Pm* promoter is mediated by the host RNA polymerase and regulated by the plasmid-encoded transcriptional activator XylS; the latter stimulates transcription in the presence of aromatic compounds that enter cells without a specific transport system, enabling use of this inducible promoter system in a variety of different organisms (33). The *Pm* promoter can yield protein expression levels as high as those obtained using a phage T7 promoter (2). Replication of the vector is supported by a mini-RK2 replicon, which consists of the vegetative replication origin *oriV* and the essential replication initiation protein TrfA. The mini-RK2 replicon has been shown to support plasmid replication in at least nine different gram-negative bacteria (37). Further, the copy number of the vector can be modified by using copy number mutants of *trfA* (2). The vector also carries a conjugal transfer origin (*oriT*) and a tetracycline resistance gene (*tetA*) for selection but lacks the *parDE* segment, which is required for plasmid stability in the absence of selection (35, 39). The latter feature facilitates curing of the donor plasmid after gene targeting.

The targetron cassette expressed from pBL1 consists of a 0.9-kb Ll.LtrB-ΔORF intron and short flanking 5′ and 3′ exon sequences (E1 and E2), with the IEP (denoted LtrA) expressed from a position just downstream of E2 (Fig. 2A) (16, 21). Induction with *m*-toluic acid results in the synthesis of a precursor RNA containing the Ll.LtrB-ΔORF intron and flanking exons, followed by *ltrA* (Fig. 2B). The LtrA protein expressed from this RNA binds to the Ll.LtrB-ΔORF intron and promotes its splicing by stabilizing the catalytically active RNA structure. It then remains bound to the excised intron lariat RNA in RNPs that promote DNA integration by the mechanism shown schematically in Fig. 2B and described in detail in reference 23. After integration into a DNA target site, the Ll.LtrB-ΔORF intron cannot be spliced or remobilized in the absence of the IEP, yielding a gene disruption. Moreover, the Ll.LtrB-ΔORF is less susceptible to nuclease degradation than is the full-length intron, leading to substantially higher integration frequencies (16).

Test of pBL1 in *E. coli*. First, we tested the pBL1 donor plasmid in *E. coli* strain HMS174(DE3), using a targetron (LacZ635s) that inserts site specifically into the *lacZ* gene, so that integration could be scored simply by blue/white screening (Fig. 1B). In initial experiments for determination of optimal conditions, targetron expression was induced at 30 or 37°C with different concentrations of *m*-toluic acid (2, 5, or 10 mM) for different times (1 h, 2 h, or overnight). The highest frequency of disruptants was obtained by using 2 mM *m*-toluic acid for 1 h at 37°C. Under these conditions, the LacZ635s

targetron expressed from pBL1 gave 10% disruptants, compared to 7% for the same targetron expressed in *E. coli* strain HMS174(DE3) from donor plasmid pACD2X by using a T7*lac* promoter (data not shown). Insertion of the targetron at the correct site in the *lacZ* gene was confirmed by sequencing the PCR products (not shown). Thus, pBL1 appears to be at least as efficient as a T7-based targetron expression plasmid for gene targeting in *E. coli* and has the advantage of not requiring introduction of a gene encoding T7 RNA polymerase.

Gene targeting in *P. aeruginosa*. To test whether pBL1 could be used for gene targeting in *P. aeruginosa*, we selected two candidate genes, *pqsA* and *pqsH*, which encode enzymes involved in producing PQS (2-heptyl-3-hydroxy-4-quinolone) (12). The latter is a signaling molecule that controls multiple virulence factors and is required for synthesis of the pigmented compound pyocyanin, enabling visual identification of disruptants (9, 31).

To construct targetrons that insert within *pqsA* and *pqsH*, we used the computer algorithm to identify potential Ll.LtrB insertion sites and selected a single target site for each gene (PqsA position 621a and PqsH position 108s) from among multiple potential target sites, based on computer ranking and E values (for PqsA621a, score 9.9, E value 0.023; for PqsH108s, score 8.55, E value 0.072) (30). pBL1-based donor plasmids containing targetrons PqsA621a and PqsH108s, which are targeted to insert into the selected sites, were constructed by a two-step PCR (Fig. 2C), using primers designed by the algorithm to modify the intron's EBS1, EBS2, and δ sequences to base pair optimally to DNA target site sequences IBS2, IBS1, and δ′. The IBS1 and IBS2 sequences in the 5′ exon of the donor plasmid were also modified in the same PCR to be complementary to the intron RNA's retargeted EBS1 and EBS2 sequences for efficient splicing from the precursor RNA synthesized from the donor plasmid (see Materials and Methods). The target sites for targetrons PqsA621a and PqsH108s and their predicted base-pairing interactions with the DNA target sequence are shown in Fig. 1B.

The donor plasmids containing targetrons PqsA621a and PqsH108s were electroporated into *P. aeruginosa* PAO1, and the transformants were grown to early log phase in the presence of tetracycline and induced with *m*-toluic acid. The cells were then serially diluted and plated on LB agar containing tetracycline. After incubation of the plates overnight at 37°C, disruptants were identified by colony PCR screening, using primers flanking the predicted insertion site in the target gene. The optimal conditions for induction of the PqsA621a targetron in *P. aeruginosa*, determined as described above for *E. coli*, were 2 mM *m*-toluic acid and induction overnight at 30°C. Under these conditions, 7/15 (47%) colonies had the desired disruption, which was detected by colony PCR (Fig. 3A, left panel) and confirmed by sequencing the PCR products (not shown). Induction for only 1 or 2 h gave lower frequencies (15 to 20%), and induction at 37°C gave frequencies 5- to 10-fold lower than that at 30°C (data not shown).

For PqsH108s, only 1 of the 96 colonies screened by PCR had the desired disruption (Fig. 3B, left panel), which was again confirmed by sequencing the colony PCR product (not shown). The much lower targeting efficiency of PqsH108s than of PqsA621a was not predicted by their computer ranking and could be due to a number of factors (see Discussion). As expected, both the *pqsA* and the *pqsH* disruptants were unable to synthesize pyocyanin (8, 12), a blue pigment, which appears green against the yellow background of the BHI agar plates (Fig. 3C).

To assess the specificity of targetron integration, we carried out Southern hybridizations on DNA isolated from randomly chosen disruptants, after curing the donor plasmid by overnight growth in liquid culture in the absence of selection (see Materials and Methods). All of the disruptants analyzed showed just one band of the size expected for insertion at the desired site in the *pqsA* or *pqsH* gene, with no additional bands due to nonspecific insertion (Fig. 3A and B, right).

Gene targeting in *A. tumefaciens*. To test gene targeting in *A. tumefaciens*, we chose the *aopB* gene, which encodes an outer membrane protein involved in crown gall tumorigenesis in host plants (20). A pBL1-based donor plasmid containing targetron AopB567a (Fig. 1B), which is targeted for insertion at position 567a within *aopB*, was designed and constructed as described above. The donor plasmid was then electroporated into *A. tumefaciens* C58, and cells were grown to early log phase in MG/L medium containing tetracycline, induced with 5 mM *m*-toluic acid for 3 h at 30°C, and plated on MG/L agar with tetracycline. In this case, 6/48 colonies screened by PCR with primers flanking the insertion site showed a single band corresponding to the disrupted gene, and at least 10 additional colonies contained a mixture of wild-type and disruptant genes (Fig. 4, left panel, shows a subset of the PCR results). The integration frequency did not increase significantly when induction was overnight instead of 3 h. The mixed colonies obtained with AopB567a presumably reflect integrations resulting from leaky expression of the targetron after plating and could be resolved readily by restreaking. Again, the correct disruption was confirmed by sequencing the colony PCR products (not shown), and for two randomly chosen disruptants cured for the donor plasmid Southern hybridization with an intron probe showed a single band of the size expected for insertion at the selected target site, with no additional bands due to nonspecific insertions (Fig. 4, right panel).

We also tested a targetron (ChvI609a) against the *A. tumefaciens chvI* gene, which encodes an *E. coli phoB* regulatory gene homolog required for virulence (24). In this case, 15% (7/48) of the screened colonies had the correct disruption, which was again detected by PCR and confirmed by sequencing the PCR product (Fig. 1B and data not shown).

Gene targeting via conjugation. Many bacteria are difficult to transform, but exogenous DNA can be introduced readily by conjugation (15, 44). The pBL1 donor plasmid contains the conjugal transfer origin, *oriT*, enabling transfer of the plasmid by conjugation between *E. coli* and other bacteria (2). Figure 5 shows results for experiments in which the pBL1-based donor plasmids containing targetron PqsA621a or AopB567a (see above) were electroporated into *E. coli* strain S17.1 and then conjugated into *P. aeruginosa* or *A. tumefaciens*, respectively (see Materials and Methods). Cells that acquired the donor plasmid by conjugation were grown in liquid culture, induced as described above for each organism, and plated on LB agar containing tetracycline plus chloramphenicol (*P. aeruginosa*) or MG/L agar containing tetracycline plus ampicillin (*A. tumefaciens*). For both organisms, the targetron introduced by conjugation had targeting efficiencies similar to those introduced by

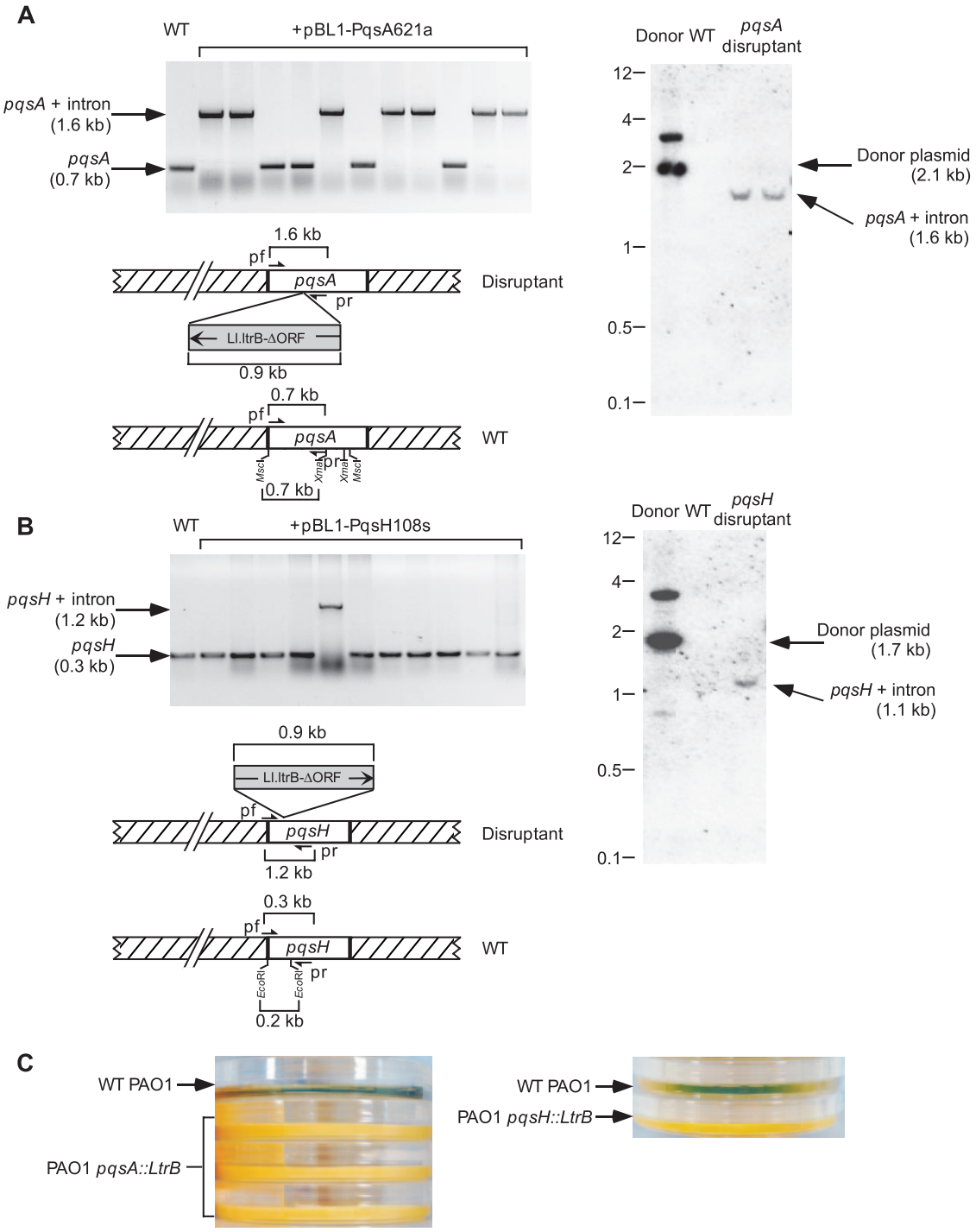

FIG. 3. Disruption of the *P. aeruginosa pqsA* and *pqsH* genes by use of targetrons expressed from pBL1. (A and B) Disruption of the *pqsA* and *pqsH* genes in *P. aeruginosa* PAO1. The left panels show colony PCR of untransformed *P. aeruginosa* wild-type (WT) PAO1 and a sampling of colonies screened for disruptants obtained with each targetron. The right panels show Southern blots of donor plasmids (left lanes) and genomic DNAs from WT PAO1 and from randomly chosen *pqsA* and *pqsH* disruptants after curing of the donor plasmid (right lanes). DNAs were digested with MscI plus XmaI (*pqsA*) or MscI plus EcoRI (*pqsH*), run in a 0.8% agarose gel, blotted to a nylon membrane, and hybridized with a ^{32}P-labeled intron probe. The numbers to the left of the gel indicate size markers in kilobases. The schematic at the bottom shows the *pqsA* and *pqsH* genes, with (top) and without (bottom) the inserted targetron, including the locations of primers p_f and p_r, used to detect targetron insertion by colony PCR, and the restriction sites used for Southern hybridization. (C) Phenotype of *pqsA* and *pqsH* disruptants. The disruptants were streaked on a BHI agar plate and incubated at 37°C overnight. The blue pigment pyocyanin appears green against the background of the agar plates.

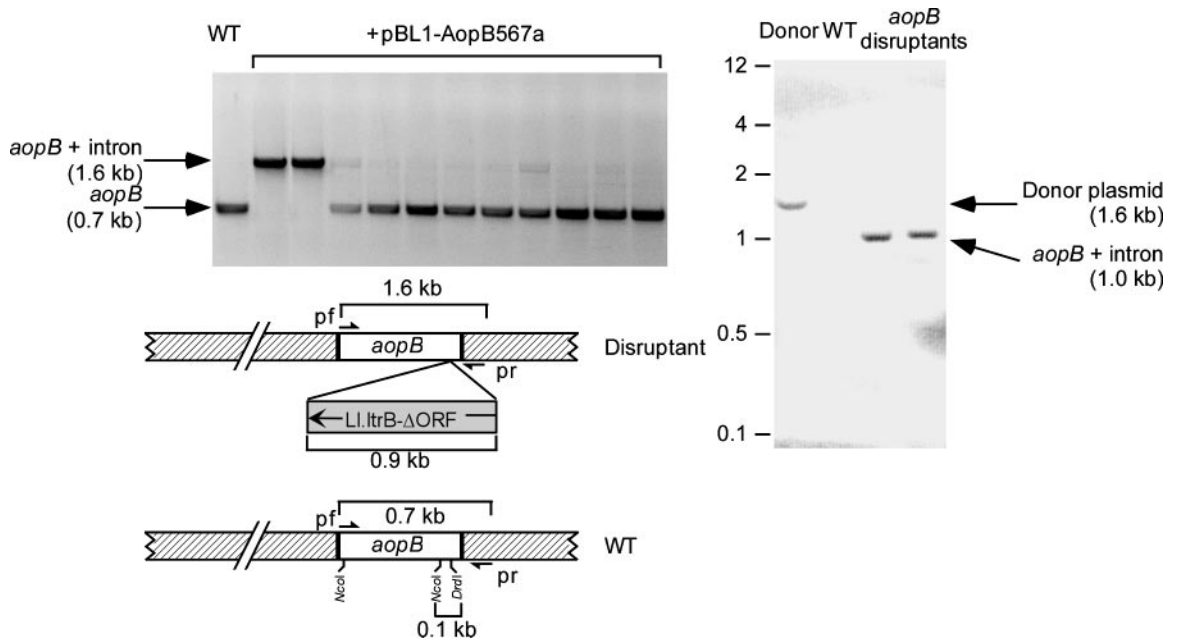

FIG. 4. Disruption of the *A. tumefaciens aopB* gene by use of a targetron expressed from pBL1. The left panels show colony PCR of *A. tumefaciens* untransformed wild-type (WT) strain C58 and a sampling of colonies screened for disruptants with the AopB567a targetron. The right panels show Southern blots of donor plasmids (left lanes) and genomic DNA from WT C58 and from randomly chosen *aopB* disruptants after curing of the donor plasmid (right lanes). DNAs were digested with DrdI, EcoRI, and NcoI, run in a 0.8% agarose gel, blotted to a nylon membrane, and hybridized with a ^{32}P-labeled intron probe. The numbers to the left of the gel indicate size markers in kilobases.

electroporation (for PqsA621a, 7/12 [58%] by conjugation compared to 7/15 [47%] by electroporation; for AopB567a, 4/48 [8%] by conjugation compared to 6/48 [12%] by electroporation) (Fig. 1 and 5A and B). These findings demonstrate that conjugation is an efficient means of introducing targetrons for gene targeting in both *P. aeruginosa* and *A. tumefaciens* and suggest that this will also be the case for other bacteria.

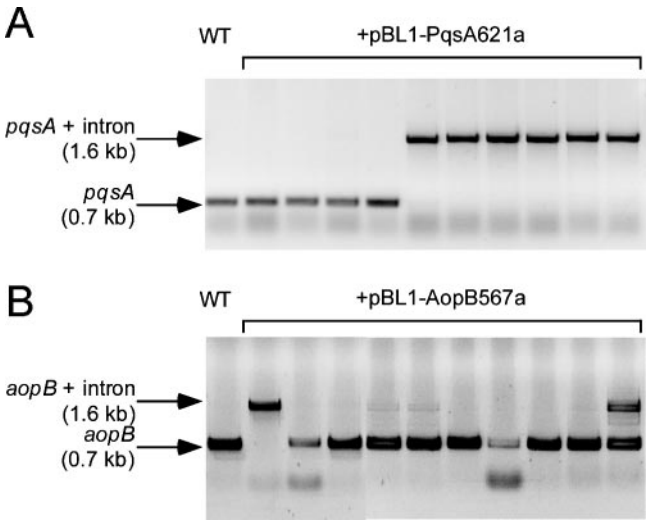

FIG. 5. Gene targeting in *P. aeruginosa* and *A. tumefaciens* by using targetrons introduced via conjugation of pBL1. Colony PCR of wild-type (WT) *P. aeruginosa* PAO1 and a sampling of potential *pqsA* disruptants (A) and WT *A. tumefaciens* C58 and a sampling of potential *aopB* disruptants (B). In both cases, the conjugal transfer frequency was 10^{-3} to 10^{-4} per donor cell. In panel B, several isolates show mixtures of disrupted and undisrupted *aopB* genes (particularly prominent in the right-hand lane). Such mixtures presumably reflect intron integration during or after plating and were readily resolved by restreaking.

DISCUSSION

We show here that targetrons expressed via an *m*-toluic acid-inducible promoter (*Pm*) from a broad-host-range donor plasmid (pBL1) containing a mini-RK2 replicon can be used for efficient gene targeting in *E. coli*, *P. aeruginosa*, and *A. tumefaciens*. The RK2 plasmid is a large (60-kb), naturally occurring, self-transmissible IncPα plasmid (42), which can replicate in at least 33 different gram-negative bacteria and 1 gram-positive bacterium (2, 26). The minimal RK2 replicon derived from this plasmid and used in pBL1 consists of *oriV* and *trfA* and has been shown to support plasmid replication in at least nine gram-negative bacteria (*Acinetobacter calcoaceticus*, *A. tumefaciens*, *Azotobacter vinelandii*, *Caulobacter crescentus*, *E. coli*, *P. aeruginosa*, *Pseudomonas putida*, *Rhizobium meliloti*, and *Rhodopseudomonas sphaeroides*) (37). Based on the properties of the mini-RK2 replicon and the inducible *Pm* promoter, we anticipate that pBL1 will be useful for targetron expression in many gram-negative and possibly some gram-positive bacteria. We note that the optimal conditions for targetron induction from pBL1 differ for each of the three bacteria tested here and will need to be determined in each new case by varying induction time, temperature, and *m*-toluic acid concentration. The ability to use targetrons for gene targeting in *A. tumefaciens* may facilitate the modification of strains, Ti plasmids, and transferred DNAs (T-DNAs) for plant genetic engineering (14).

The present work also shows for the first time that targetrons introduced by conjugation function as efficiently for gene targeting as those introduced by electroporation. This finding was expected because the wild-type Ll.LtrB intron is found in a conjugal element and was shown previously to retrohome to the wild-type target site and retranspose to ectopic sites when transferred by conjugation between different *L. lactis* strains or between *L. lactis* and *Enterococcus faecalis* (1, 27, 38). The ability to introduce targetrons by conjugation enables their use in organisms that are not amenable to the introduction of foreign DNA by transformation or electroporation.

As found previously for other bacteria, a targetron based on the Ll.LtrB-ΔORF intron functions efficiently enough in *P. aeruginosa* and *A. tumefaciens* to detect site-specific integrations simply by colony PCR screening. In *E. coli* and *L. lactis*, it is has also been possible to select integrants by using targetrons carrying genetic markers, including retrotransposition-activated markers, inserted in malleable regions of intron domain IV (11, 30, 32, 47). A cautionary note is that while mobile group II introns containing a Kan^r retrotransposition-activated marker gene (also known as a retrotransposition indicator gene) function well when expressed in *E. coli* with a T7 promoter or in *L. lactis* with a nisin-inducible promoter (5, 6, 19), such targetrons did not function well in *S. aureus* with a cadmium-inducible promoter or in *P. aeruginosa* with the *Pm* promoter in the pBL1 plasmid constructed here (J. Yao and A. M. Lambowitz, unpublished data). The reasons for these differences are unknown, but one possibility is that they reflect differences in the RNA polymerases used in different hosts for targetron expression, particularly in the processivity required to transcribe the long, highly structured intron RNA containing the genetic marker. In nature, the inability of some host RNA polymerases to efficiently transcribe full-length mobile group II introns encoding the reverse transcriptase may limit the spread of these introns and could account at least in part for their preferential insertion into sites outside bacterial genes (7). In organisms that lack a suitable endogenous RNA polymerase, genetically marked targetrons might still be employed, either by using a small marker gene, e.g., the trimethoprim resistance gene, or by introducing T7 RNA polymerase for targetron expression (47).

For the five genes in three different bacteria tested in the present work, the computer algorithm used for target site selection and targetron design gave targetrons that are inserted at frequencies of 1 to 58% of screened colonies without selection, with no failures. Similar high frequencies of gene targeting were observed for targetrons designed by the same algorithm in *E. coli*, *L. lactis*, *C. perfringens*, and *S. aureus* (3, 11, 30, 45). Nevertheless, the algorithm does not always reliably predict relative integration efficiency, as evidenced here by the much lower than expected integration frequency of targetron PqsH108s compared to that of PsqA621a, even though both targetrons were scored highly by the algorithm. This is so because the algorithm uses a simple probabilistic model in which each position in the target site is assigned a weighted value corresponding to the nucleotide frequency in a database of verified Ll.LtrB intron target sites, and the ranking of the target site is determined as the product of these frequencies (30). As discussed previously, the database upon which the algorithm was based was not sufficiently large to reliably predict covariations between different target site positions. Additionally, decreased efficiency could result from suboptimal base-pairing interactions with the intron RNA, deleterious effects of nucleotide substitutions on intron RNA activity, occlusion of some target sites by proteins, or contributions of other factors, such as duplex stability or higher-order DNA structure (30). Recent studies showed that integration of Ll.LtrB intron RNPs results in bending of the target DNA, and computational analysis suggested that DNA bendability in addition to sequence may influence DNA integration efficiency (29). We anticipate that the algorithm will continue to improve as larger databases become available and more information about other factors is incorporated.

In summary, our results establish that broad-host-range vectors can be used to express targetrons for gene targeting in multiple species of bacteria. The broad-range vector in the present work was developed for gram-negative bacteria, and we now look forward to the development of equally efficient broad-host-range vectors for gram-positive bacteria.

ACKNOWLEDGMENTS

We thank Svein Valla (Norwegian University of Science and Technology) for vector pJB866, Eugene Nester (University of Washington) for *A. tumefaciens* strain C58 and target site suggestions, and Marvin Whiteley (University of Texas at Austin) for *P. aeruginosa* strain PAO1 and target site suggestions.

This work was supported by NIH grant GM37949 and Welch Foundation grant F-1607.

Group II intron gene targeting technology is subject to patents licensed by the Ohio State University and the University of Texas at Austin to InGex, LLC, and rights to sell research tools based on the technology are sublicensed to Sigma-Aldrich. A.M.L. is a minority equity holder in InGex, LLC, and both authors potentially share in royalties paid to Ohio State University and the University of Texas at Austin.

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